学位論文要約（博士（理学・工学））

論文著者名　鴨志田　一

論文題名：Heteronuclear mutidimensional NMR studies of proteins in cultured human cells
（邦題）：異種核多次元 NMR を用いたヒト培養細胞内蛋白質の解析（英文）

In vivo observations of 3D structures, structural changes, folding stability, dynamics or interactions of proteins are essential for the explicit understanding of the structural basis of their functions inside cells. In-cell NMR [1] is currently the only approach that can provide structural information of proteins inside cells at atomic resolution.

Although in-cell NMR was first established using *E.coli* recombinant expression systems, further applications to proteins in human cultured cells have raised the prospect of providing direct information for medical and pharmaceutical applications, e.g. screening of drug candidates. Unless producing in the cells, the proteins of interest are incorporated into human cultured cells from outside by various methods utilising cell-penetrating peptides [2], streptolysin O [3], and electroporation [4]. However, the maximum achieved protein concentration in cells (e.g. ~30 μM when using cell-penetrating peptides) is, at least for globular proteins, too low to obtain backbone NMR resonances assignments exclusively from in-cell NMR data, thus limiting the application to the cases where the assignments of in-cell NMR spectra can be transferred from those obtained in vitro. If the proteins of interest experience large structural changes in cells, large chemical shift changes must be expected, which would hinder the transfer of in vitro assignments to in-cell spectra. Theoretically, the resonance assignment can be done by repeating in-cell NMR experiments using a series of selectively labelled samples which are produced by, e.g. residue-selective stable-isotope enrichment using umber-suppressor systems. Nevertheless, for wider applications, more general assignment strategies using uniformly labelled-proteins are awaited.

As model proteins, I selected four small proteins, human calbindin D_{9k} with P47M mutation and an extra Cys residue at the C-terminus (80 a.a), human ubiquitin with L8A/I44A/V70A mutations (76 a.a), *T. thermophiles* HB8 TTHA1718 (66 a.a), and *Streptococcus* protein G B1 domain (57 a.a, henceforth referred to as GB1). Cultured HeLa.S3 cells, which has better reputation in the viability in the NMR tubes, were used as host cells, and the electroporation was selected as the protein incorporation method. By optimising the parameters for

electroporation high resolution 2D ^{1}H-^{15}N SOFAST-HMQC spectra were obtained for all four model proteins with relatively good sensitivity.

Next, GB1 was selected for the trial triple-resonance NMR experiments. 3D HNCA experiment (conventional water-flip-back version) was performed for ^{13}C/^{15}N-labelled GB1 in HeLa.S3 cells. The data were acquired by employing the nonlinear sampling scheme (total experimental time of ~6 hrs). The viabilities of the HeLa.S3 cells after ~7 h of NMR experiments was confirmed to be 85±8 % by trypan blue exclusion test. The obtained HNCA spectrum showed disappointing quality. Crowded intracellular environment makes the rotational correlation time of proteins longer mainly because of the increased viscosity. The situation causes the $^{13}C^{\alpha}$ transverse relaxation time (T_2) shorter, which is presumably the reason of such a low sensitivity in the HNCA spectrum. I took two strategies to overcome the problem: (1) employing BEST-HNCA or BEST-TROSY-HNCA pulse sequence with selective ^{1}H pulses, which enables shorter recycling delay in each scan, thus providing approximately 30 % increase in signal-to-noise ratio in the unit measurement time, and (2) employing TROSY-HNCA pulse sequence on perdeuterated proteins, which have much slower $^{13}C^{\alpha}$ T_2 relaxation because of the much smaller gyromagnetic ratio of deuteron when compared with that of proton. The 3D BEST-HNCA and 3D BEST-TROSY-HNCA spectra were acquired for ^{13}C/^{15}N-labelled GB1 in HeLa.S3 cells, while 3D TROSY-HNCA spectra were acquired for ^{2}H/^{13}C/^{15}N-labelled samples. Recycling delays of 0.5, 0.5 and 1.5 s were used for the BEST-, BEST-TROSY and TROSY-HNCA experiments, respectively.

Although both modifications provided improved sensitivity when compared to conventional HNCA, TROSY-HNCA spectra of ^{2}H/^{13}C/^{15}N-labelled GB1 showed the best sensitivity in the unit measurement time. By analysing the TROSY-HNCA spectra in combination with 3D TROSY-HN(CO)CA, TROSY-HNCO and T1`ROSY-HN(CA)CO spectra, which were measured on ^{2}H/^{13}C/^{15}N-labelled GB1 in HeLa.S3 cells by employing the same strategy, backbone $^{1}H^{N}$, $^{13}C^{\alpha}$, ^{13}C' and ^{15}N resonances were assigned exclusively from in-cell NMR spectra for 55 out of 57 residues of GB1 in living HeLa.S3 cells. To the best of my knowledge, this is the world's first achievement for proteins in cultured human cells, suggesting that further NMR analyses based on the *ab initio* backbone resonance assignments, such as side-chain resonance assignments, collection of distance restraints, monitoring various intracellular events, are feasible.

References

[1] Serber, Z. et al. *J. Am. Chem. Soc.* 123, 2446-2447 (2001); [2] Inomata, K. et al. *Nature* **458**, 106 (2009); [3] Ogino, S. et al. *J. Am. Chem. Soc.* **131**, 10834 (2009); [4] Danielsson, J. et al. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 12402 (2015)